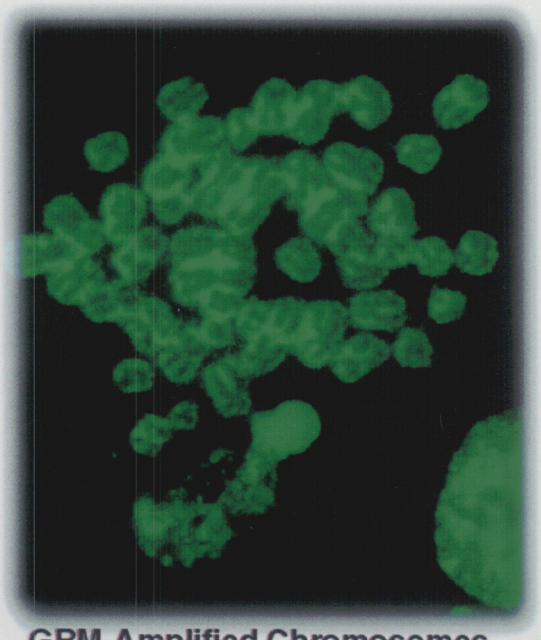
UCRL-ID-142409

2001 R&D 100 Awards Entry Form

Gene Recovery Microdissection (GRM)

A Process for Producing Chromosome Region-Specific Libraries of Expressed Genes

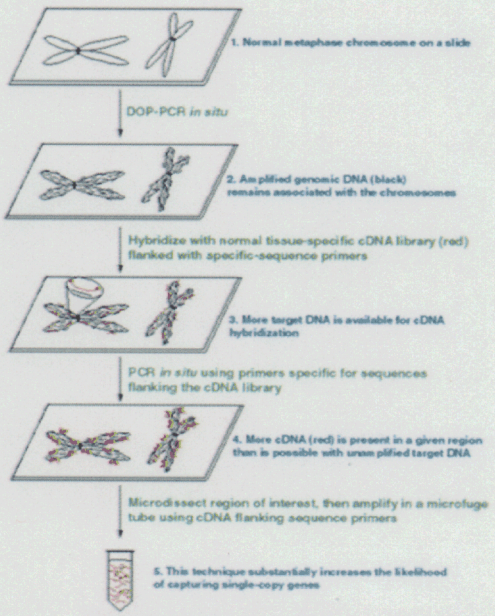


GRM-Amplified Chromosomes

A unique and cost-effective process for producing chromosome region-specific libraries of expressed genes. It accelerates the pace, reduces the cost, and extends the capabilities of functional genomic research, the means by which scientists will put to life-saving, life-enhancing use their knowledge of any plant or animal genome.

This work was performed under the auspices of the U.S. Department of Energy by the University of California, Lawrence Livermore National Laboratory under Contract No. W-7405-Eng-48.

Allen T. Christian
Matthew A. Coleman
James D. Tucker



GRM Process Flowchart

2001 R&D 100 Awards Entry Form

1. Submitting Organization

Organization:	Lawrence Livermore National Laboratory (LLNL)
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Country:	USA
Submitter's Name:	Allen T. Christian
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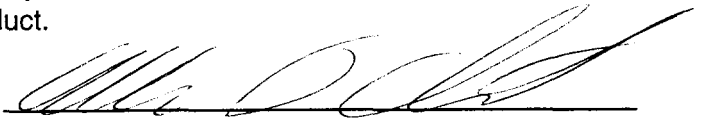
Email:	christian4@llnl.gov

AFFIRMATION: I affirm that all information submitted as a part of, or supplemental to, this entry is a fair and accurate representation of this product.

Submitter's signature: 

2. Joint entry with:

Organization: None

3. Product name:

Gene Recovery Microdissection (GRM)

4. Briefly describe what the entry is:

GRM is a process for amplifying DNA fixed to a medium and can be used to produce chromosome region-specific libraries of expressed genes of virtually any plant or animal species for use in functional genomic research.

5. When was this product first marketed or available for order?

GRM was first announced in the Department of Commerce's *Commerce Business Daily* on February 7, 2000.

6. Inventor or Principal Developer:

List additional developers from all companies on a separate sheet in an appendix and check here [**X**]

Developer Name:	Allen T. Christian
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7. Product price:

If the price is proprietary, fill in the blank and also check here: [ ]

LLNL plans to market GRM as a series of licenses relating to specific products, such as gene libraries, and to the process by which they are made. Prices will be set depending on the type of product or process licensed. Three companies are currently negotiating with LLNL to license GRM.

8. Do you hold any patents or patents pending on this product?

Yes [**X**]

No [ ]

9. Product Description:

What Does It Do? Gene Recovery Microdissection (GRM) is a process used to produce libraries of all of the genes expressed in any chromosomal region of any tissue of any plant or animal species. It can also be used to clone all of the DNA in any organism, including bacteria, even those that can't be cultured. While every nucleated cell in an organism contains the same set of genes, cells in each tissue use a different subset of all the genes in the organism's genome. If one thinks of the nucleus of a cell as a library, then the chromosomes in the cell are bookshelves in that library and the genes are the books on each shelf. Each cell in a particular organism, whether it's a particular person or an individual plant, contains the same libraries, and the same sets of books. These books represent all of information (the DNA) that every cell in the body needs so that it can grow and carry out its various functions.

Not all of these genes are used, or expressed, by every cell in the body, however. For example, some processes that are particular to cells in the liver are completely unused in brain cells. So each cell type only uses some of the books in its library. Further complicating this system is the fact that less than ten percent of the DNA is actually used to make genes. This is equivalent to having most of the books in a library filled with nonsense, with only occasional passages that are important. The reason that genome projects are so expensive is that there is no good way of learning which genes are being used at any given time, and from which chromosome they came. The process that we have developed is a way to isolate all of the genes that are being used from a particular chromosome region by a specific tissue at any point in time.

No longer will it be necessary to sequence the entire genome of every species just to find its genes. Using GRM, investigators can conduct a search for relevant genomic information on species of interest without having to go to the effort of sequencing the entire genome of that species, an expensive and time-consuming process that is beyond the capabilities of all but the most well-funded and sophisticated laboratories.

How does it do it? GRM works by combining cytogenetics and genomics, two disciplines that study DNA structure and sequence in significantly different ways, with chromosome microdissection. The product of gene expression is messenger RNA, or mRNA. Typically, before any genetic engineering is done, the mRNA molecules are converted into more stable DNA molecules. The resulting product, called cDNA, has exactly the same sequence as the mRNA but is easier to handle. A cDNA library consists of all the genes expressed in a particular tissue from a particular species. It is, in essence, a collection of photocopied pages from the books that are in use by the cells. When the cDNA library is combined on a microscope slide with chromosomes, the cDNA molecules hybridize to the chromosomal regions corresponding to the genes, a process equivalent to reinserting the photocopies into the books from which they came. Regions of the chromosomes of interest to scientists can then be isolated, and with them the hybridized cDNA molecules, using tiny glass needles and a process called microdissection. As an aspect of their manufacture, the cDNA molecules have specific DNA sequences attached to each end, that enable polymerase-chain-reaction (PCR) amplification and sequencing following microdissection.

Although the basic technique of using microdissection to isolate genes has existed for about five years, no commercially available libraries have been generated, because of a lack of sensitivity resulting from the inefficiencies inherent in the hybridization and subsequent PCR amplification. Because genes are typically represented only once in a chromosome, a maximum of one cDNA molecule will be present for each expressed gene following microdissection. Successful hybridization, dissection and PCR amplification of a single molecule is virtually impossible. As a result, libraries made with this procedure are so incomplete as to be unusable. To continue the library analogy, it is as if one had been asked to photocopy several pages from tens of thousands of books, reinsert the photo copies in the books, and then remove only those that are filed on a

particular shelf. The possibility of failing to photocopy some pages, failing to reinsert some, and forgetting to remove others during the final isolation step makes it virtually impossible to leave the library with all of the photocopied material.

GRM overcomes this inefficiency by increasing both the number of targets available for cDNA hybridization and by increasing the total number of cDNA molecules in each region following hybridization. It does so by using PCR in situ, which is PCR done on the slide rather than in a tube, the conventional means. First, a random-primed PCR on the chromosomes on the slide prior to hybridization produces many copies of the target DNA, significantly improving the chances of cDNA hybridization. Second, following the hybridization, a second PCR amplification using primers specific for the ends of the cDNA molecules is performed, increasing the numbers of bound cDNA molecules. Instead of isolating a maximum of one cDNA molecule per expressed gene in a region, the GRM process recovers hundreds or even thousands of cDNA molecules. It is as if, instead of one copy of each book, there are fifty. Instead of one copy of each page, there are hundreds. This simple step makes the production of highly useful chromosome region-specific libraries possible.

10A. Product Competitor Methods

Although scientists have developed a number of techniques that provide partial information about gene expression and genomic location, no single technique both identifies expressed genes and determines the part of the genome that regulates their expression. For example, fluorescence in situ hybridization (FISH) allows the identification of gene regions on the chromosome, but its probes are incapable of identifying the new genomic sequence for novel genes, which is done by sequencing. Microdissection can obtain specific genes from a chromosome region, but the inefficiencies in the process, from the hybridization to the dissection to the PCR amplification of the dissected fragments, have made it necessary to dissect many fragments to obtain all or most of the genes in a specific region. Gene sequencing can provide complete gene identification and location, but not information about the specific tissue in which the gene is expressed. Genomic sequences usually do not identify chromosome location, and sequence alone cannot identify where the gene is expressed. Nonregion-specific cDNA libraries (the only sort commercially available) give information regarding the genes that are expressed in a particular tissue or under a specific condition, but they provide no information regarding chromosomal location. Also, as many as 20,000 copies of any given gene are present in any one cell, and obtaining a complete sequence for all of these is laborious and expensive.

The GRM process, however, can provide in a single step information that would otherwise require gene isolation, sequencing, and location by hybridization.

As shown in the table below, various companies provide commercially available products or processes that supply some of the capabilities of GRM, but none of them provides all of the expression, sequencing, and location information made possible by the chromosome region-specific libraries of expressed genes produced by GRM.

Table 1. Several companies provide components related to GRM-libraries.
(Yes = a product is available from that company. No = a product is not currently available from that company.)

	cDNA Libraries (Expression Data)	Gene Sequence (Sequence Data)	FISH Probes (Location Data)
Promega	Yes	No	No
Invitrogen	Yes	No	No
Origen	Yes	No	No
Ambion	Yes	No	No
Clontech	Yes	No	No
Molecular Probes	No	No	Yes
Celera	Yes	Yes	No
Incyte	Yes	Yes	No

10B. Comparison Matrix

As shown in Table 1 above, some aspects of GRM—expression, sequence, and/or location data—can be obtained using commercially available processes. However, as Table 2 below makes clear, none can provide all of the biological information needed to understand gene function that GRM provides in a single step.

Table 2. Only GRM can provide chromosome region-specific libraries of expressed genes.

Technique	**GRM**	FISH	cDNA Sequencing	DNA Sequencing
Gene localization	**X**	X		
Gene sequence	**X**		X	X
Tissue specificity	**X**	X	X	
Multiple species	**X**	X	X	X

10C. Improvements Upon Competitive Products or Technologies

GRM makes possible in one process what was previously possible only in part using multiple processes—and it does so robustly and cost-effectively. Each of the libraries takes only days to produce, and each can be copied thousands of times in a single day. This is in contrast to sequencing a genome, which can take years. GRM can produce libraries of all of the genes expressed in any chromosomal region of any tissue of any plant or animal species.

11A. Principal applications of this product.

The GRM technology has numerous applications, and is currently being used successfully in the areas of cancer research, gene expression libraries production, functional genomics, toxicology and pharmacology, immunology, developmental biology and embryology, veterinary medicine, and environmental and ecosystem sciences. Potential areas of applications include oncology and pediatric medicine, pending FDA approval.

Cancer. GRM was invented to allow researchers to identify cancer genes in chromosomal regions for which scientists yet had no genomic information. Initially, these were regions for which scientists had good evidence concerning their importance in rat mammary cancer but almost no other knowledge. To identify the genes expressed in these regions (and thus thought to be important for mammary carcinogenesis), researchers needed a quick, simple, inexpensive, and reliable method of identifying and characterizing both novel and previously known genes in any chromosomal region. All of these objectives were accomplished by GRM, and it is this application that continues to be the primary use of this technology.

Libraries of DNA sequences expressed in animals and plants. GRM can be used to generate chromosome and chromosome region-specific libraries of genes that are expressed for any tissue, normal or diseased, for any organism—all vertebrate animals, numerous invertebrate animals, and all plants—that can have their chromosomes spread on a microscope slide. Once these libraries have been produced, they can easily be placed on microarrays and made available to other investigators for more detailed analyses, including gene expression studies. GRM can thus be used to create a systematic approach to identifying genes expressed in virtually every animal and plant species of interest to humans, both in terms of medical and veterinary medical practice and in agriculture. No longer will it be necessary to sequence the entire genome of every species just to find its genes. Investigators now have the option of conducting a search for relevant genomic information on species of interest without having to go to the effort of sequencing the entire genome, an expensive and time-consuming process that is beyond the capabilities of all but the most well-funded and sophisticated laboratories. GRM delivers short (~200- to ~1000-base-pair) DNA molecules that are directly amenable to sequencing by small laboratories with only one or a few sequencing machines. Furthermore, GRM focuses on data that current genomic sequencing efforts cannot provide, namely information concerning the *expression* of genes on a tissue-by-tissue basis. This capability is critically important because information concerning tissue-specific expression of genes cannot yet be determined from DNA sequence alone.

11B. Other applications for which your product can be used.

Drug discovery. Many drugs have been developed by culturing and sequencing bacteria. Current technology requires that bacteria be grown in culture prior to sequencing in order to obtain sufficient DNA for the sequencing process. More than 95% of all bacteria cannot be grown in culture, a fact that greatly limits the process of drug discovery. GRM can be used to provide enough DNA for sequencing from one bacterium, eliminating the need for cultures and allowing all bacteria to be screened for new genes.

Functional genomics. GRM will enable investigators to identify genomic sequences that are close to expressed genes that contain DNA code for determining the tissues and environmental conditions under which genes are expressed. GRM is poised to help us interpret the vast quantities of genomic sequence data now being generated for many animal and plant species. It is precisely this interpretation that the genomic data obtained thus far currently lacks.

Genomics. GRM can be used as a preliminary step toward a full genomic analysis of an organism. By identifying genes and determining their chromosomal locations, GRM can be used to help "finish" the

genome by providing independently mapped sequences of expressed genes. These sequences are extremely useful for determining the chromosomal order (i.e., "anchoring") of the DNA sequences as they are obtained. This application has potential for saving time and money in each of the many genomic efforts, both those that are ongoing and those that will be conducted in the future.

Toxicology and Pharmacology. GRM will allow toxicologists to study cellular and organismal reactions to chemical and radiation exposure, furthering our basic understanding of the molecular mechanisms involved in responses to adverse environments, including mutagenesis and DNA damage recognition and repair. Similarly, GRM will improve the ability of the pharmaceutical industry to decipher the biological responses to drugs, with the goal of improving the safety of prescription and nonprescription medications. Researchers in the pharmaceutical and toxicology industries will be able to determine the molecular mechanisms of drug responses by learning which genes undergo changes in their expression as a result of drug therapy. It is also possible that this information could be coupled with GRM applications in oncology in order to customize drug choice, dosage, and scheduling. Please see the attached letter from Dr. C. Sid Aaron, Pharmacia and Upjohn Company, for his positive response to GRM technology.

Immunology. Disease resistance in humans and animals is provided by the immune system, which attacks invading organisms using proteins made by genes that are produced from combinations of small numbers of other genes. Scientific understanding of the processes involved in immunological reactions would be improved by using GRM to characterize the gene combinations and the gene products important to this essential body function.

Developmental biology and embryology. One of the deep mysteries of modern biology concerns the biological mechanisms involved in embryonic and fetal development. This field is currently the subject of intense investigation both in the U.S. and abroad. By performing GRM on fetal tissue samples from animals, investigators should be able to determine the cascade of genetic and cellular events involved in mammalian growth and development. The potential ramifications of this application on our understanding of human health and biology are so significant that they cannot yet be completely comprehended.

Agriculture. Little or nothing is known about the genomes of numerous plant species, the production of which is worth many billions of dollars annually. GRM will help remedy this lack of knowledge by providing an affordable way to identify important genes and determine their functions. This knowledge may be used to improve the protein, carbohydrate, and vitamin content of food crop species important to our planet's rapidly growing human population.

Veterinary medicine. Animals play essential roles in providing for human health and well-being. In addition to providing food and companionship, they are also used in research to address questions that are important to human health but cannot for ethical reasons be addressed using people. Improving the health of essential animal species such as cattle, sheep, pigs, horses, chickens, dogs, cats, rats, and mice will have indirect but tangible benefits on humans. GRM provides a means of improving the health and welfare of essential animal species by providing a basic tool to enhance genomic research. GRM may also be used to improve our understanding of the role of gene expression in animal disease processes—for example, bovine spongiform encephalitis, or mad cow disease, which is currently a major problem in Britain and appears to be spreading throughout Europe.

Environmental and ecosystem sciences. Protecting endangered species is a major component of environmental policy in the U.S. and abroad. Unfortunately, the genomes of most endangered organisms may never be sequenced because of the large number of such species and the prohibitive costs of genomic analysis. However, GRM offers the possibility of identifying and characterizing the expressed

genes, both known and novel, in a cost-effective manner for those species whose value to human health cannot be determined without careful genetic appraisal.

11C. List all potential applications. Indicate why they are not now feasible.

The following application of GRM is entirely feasible but would require approval by the Food and Drug Administration prior to their use in medicine.

Oncology. GRM has potential for use as a clinical diagnostic tool. Cells in tumors are widely known to be heterogeneous with respect to their genetic material and behavior. For example, many solid tumors have cells that invade nearby tissues or that break away from the body of the tumor and spread to other sites (i.e., metastasize). At the present time, many of the genes involved in these processes are unknown, but in the not-too-distant future, these genes will have been identified (perhaps using GRM in a research mode) with the result that their involvement in individual tumors can be determined. Understanding and characterizing the genes that are actively involved in tissue invasion and metastasis in a specific patient's tumor may help clinicians determine and individualize proper courses of treatment. GRM may help this process by providing information concerning the genes expressed in specific regions of tumors.

12. Why Should This Product Win an R&D 100 Award?

Private industry and the governments of several countries have spent billions of dollars over many years to sequence the human genome. More money and time are currently being spent to sequence the genomes of a host of different organisms. The same companies and governments that sequenced the human genome and are sequencing other plant and animal genomes are now preparing to enter the field of *functional* genomics—elucidating the meaning of the DNA sequence, which is where the practical life-saving and life-transforming uses of genomic knowledge begin.

The key to functional genomics is to determine where a gene is located on a chromosome and in which tissues it is expressed and at what levels. Finally, the expressed gene must be cloned many times over in order to produce the proteins that are the starting point of the functional genomic research. In the past, this process was done using a series of expensive and time-consuming steps.

Gene Recovery Microdissection can accomplish the initial steps of functional genomic research—location, expression, and cloning— simultaneously on a simple microscope slide, thereby providing an enormous advantage to researchers in the race to unlock the secrets of plant and animal genomes. Using GRM, investigators now have the option of conducting a search for relevant genomic information on species of interest without sequencing the entire genome of that species, an expensive and time consuming process that is beyond the capabilities of all but the most well-funded and sophisticated laboratories.

ORGANIZATION DATA

13. Chief Executive Officer of submitting company (corporate or university president, government research center director, etc.):

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14. Contact person to handle all arrangements on exhibits, banquet, and publicity:

Name:	Darlene Horne
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15. To whom should reader inquiries about your product be directed?

Name: Allen T. Christian
Position: Senior Biomedical Scientist
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Appendix A: Supporting Documents

3. **List of co-developers**
4. **Letters of support**
5. **Opportunity release from Lawrence Livermore National Laboratory**
6. **Papers and abstract from invited presentation on GRM.**

A1) Co-developers:

Developer Name:	Matthew A. Coleman
Position:	Senior Biomedical Scientist
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Developer Name:	James D. Tucker
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LifeBeam Technologies, Inc.

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January 16, 2001

To: Dr. Matthew Coleman, Lawrence Livermore National Laboratory
From: Terence S. Russell, President, LifeBeam Technologies, Inc.
Re: R&D 100 Awards

Dear Dr. Coleman:

I am writing this letter in support of your application for an R&D 100 award for Gene Recovery Microdissection (GRM). We believe that the GRM technology developed by you and Drs. Christian and Tucker is a fundamental technology platform that has the potential to act as the core of many exciting commercial applications, especially in the genomics marketplace.

As you know LifeBeam Technologies, Inc. is an early stage biotechnology company that is seeking to develop and commercialize a new, ultra-fast technology for DNA sequencing. In order for us to provide the greatest value to our end users, we also have an aggressive technology acquisition program that focuses on systems that generate valuable genomic information. This information (e.g. gene localization and expression data) typically complements raw sequence data. We believe that GRM is a perfect example of just such a technology platform.

LifeBeam Technologies, Inc. is particularly interested in developing a number of commercial applications based on GRM such as whole-chromosome gene-screening panels for multiple organism types and chromosome region-specific gene screening panels. It is our understanding, based on initial market research, that there is significant commercial demand for such applications. Our research indicates that we can develop model organism (rat, mouse, etc.) gene-screening panels, based on GRM technology, that could yield end-user cost savings of greater than 50% and time savings of greater that 90% when compared to current commercial solutions. Finally, for a number of other commercially valuable organisms, we would be able to generate powerful "one-tube" genomic mapping solutions. These applications alone would enormously speed the work of researchers in the pharmaceutical, agricultural, and biotechnology industries.

Needless to say we will be pursuing our interest in GRM. We wish you the best of luck in this contest and in the future development of such an important and exciting technology.

Sincerely,

Terence S. Russell

Terence S. Russell, Ph.D.

President
LifeBeam Technologies, Inc.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
NATIONAL HEALTH AND ENVIRONMENTAL EFFECTS
RESEARCH LABORATORY
RESEARCH TRIANGLE PARK, NC 27711

January 15, 2001

OFFICE OF
RESEARCH AND DEVELOPMENT

To Whom It May Concern:
FAX 9-1-925-424-3130

This is a letter of support for Dr. James Tucker for consideration of his development of gene-recovery microdissection (GRM) for an R&D 100 award.

Dr. Tucker and his colleagues have made important advances over the years in the general field of molecular cytogenetics. However, their most recent work is a truly important step forward in this area. Specifically, they have now developed technology, GRM, that permits the application of molecular cytogenetics in ways not possible previously.

The development of GRM permits the construction of customized chromosome region-specific cDNA libraries from any tissue and any plant or animal species. The general applicability of such an invention is obvious. As genomics research proceeds, the ability to construct such libraries is critical to understanding and exploring which genes are expressed along a segment of a chromosome in a specific tissue or organism at a specific time. This elevates gene expression studies to a level not generally possible until now. Such a method will enable chromosomal regions to be examined in detail to see if genes within a contiguous segment are expressed , perhaps coordinately, not only in one species but in essentially any eukaryotic species of interest. This invention is already permitting the Tucker group at LLNL to identify genes expressed in specific tissues and chromosomal regions of the rat, one of the most used organisms in toxicology and other biomedical research.

This invention also enables one to identify genes that are expressed after exposure to drugs or environmental chemicals or radiations. This is, perhaps, the most immediately practical application of this invention, especially by pharmaceutical companies. Again, this methodology is generally applicable. Thus, with minor modifications in primers, etc., the method can be used in a wide variety of organisms, including humans. The ability to detect altered gene expression after chemical or radiation exposure is an essential feature of "molecular medicine" or the new field of genomic pharmacology, where gene expression after drug treatment is used to identify the biochemical pathways associated with the exposure–and possibly, the disease. Having a generally applicable method by which to do this will be an important contribution to drug development.

In terms of basic research, an exciting application of GRM is for the identification of syntenic regions of chromosomes (i.e., homologous regions) among species and mapping genes within those regions. This will be especially useful for species that have not yet been so fortunate as to have their genome sequenced fully. Thus, using information on gene composition in a syntenic region of a chromosome that is well characterized in one species, this invention will permit

comparison to a probable syntenic region in another organism. Eventually, such information will have applied utility in a variety of fields, but the most immediate value will be in terms of understanding the evolutionary relatedness of chromosomes among species.

Finally, among other things, GRM will enable one to identify quickly new genes (open reading frames) without the labor of sequencing large stretches of genomic DNA. This ability has a wide range of applications, from basic to applied research. Again, the general applicability of the method makes it a versatile and adaptable technique that can be used in an array of organisms to address different types of research questions.

I am pleased to have the opportunity to comment on the invention of GRM, and I trust that this invention will be considered favorably for an R&D 100 award this year. Thank you.

Sincerely yours,

David DeMarini

David M. DeMarini, Ph.D.
Research Genetic Toxicologist

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12 January 2001

Dr. James D. Tucker
Biology and Biotechnology Research Program
P. O. Box 808, L448
Lawrence Livermore National Laboratory
Livermore, CA 94551

Dear Dr. Tucker,

Subject: Letter of Support for R&D 100 application

I have reviewed the manuscript you provided that describes Gene Recovery Microdissection. This technique is indeed interesting and deserves widespread recognition.

You have identified an important need in this era of high throughput sequencing, namely, the need to identify genes of interest without sequencing the entire genome. It is particularly clever to be able to work from single chromosome regions and develop useable libraries for study of expression and genetic damage. I applaud your work.

Specifically, I think this method will greatly assist in identifying both known and unknown genes in specific regions of the chromosome with heretofore-unavailable selectivity. It is particularly important as you point out that the utility for producing region specific cDNA libraries with any tissue or animal will be facilitated. In my opinion this method should revolutionize the way future gene mapping studies, particularly in species with poorly characterized genomes, will be done. Furthermore, the area of greatest application may be in the study of gene expression in response to physical or chemical agents.

In summary, I fully support your application for the R&D 100 Award. This novel method of genome study and dissection will be an important contributor to understanding of the genome.

Sincerely,

Dr. Sid Aaron

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Mutation Research

International Journal on Mutagenesis, Chromosome Breakage and Related Subjects

MR

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January 22, 2001

To Whom it May Concern:

I am writing to provide my enthusiastic support and endorsement for the research agenda now underway in the laboratory of Dr. James Tucker and his colleagues. I have heard Dr. Tucker make several presentations involving his use of comparative genomic hybridization protocols (CGH) and the use if these methods to examine chromosomal abnormalities induced by various carcinogens is superb science.

The pattern of chromosome-region loss from carcinogens can provide clearly distinguishable signatures for given carcinogens. In his laboratory Dr. Tucker and his group have been successfully using the gene recovery microdissection method (GRM) to produce chromosome region-specific cDNA libraries. In a recent manuscript submitted to *PNAS* Dr. Tucker's group confirmed the utility of GRM by reporting that expressed genes at rat chromosome *1p12-q31* were isolated, mapped and demonstrated to correspond to known syntenic regions in human and mouse chromosomes. I believe that the GRM methodology is suitable for studies targeted towards species with still-to-be-defined genomes. Such studies will clearly provide a critical link between cytogenetic and genomic analyses.

I support this line of research fully and explicitly. In my capacity as Editor-in-Chief of *Mutation Research* I would rate the quality, ingenuity and intent of this line of research in the highest levels of importance for our ultimate understanding of molecular mechanisms involved in mutagenesis and carcinogenesis. Should Dr. Tucker and his colleagues elect at some point to use *Mutation Research* as a vehicle for submission of some of the results of his work you can be assured we will review and process the manuscripts with the highest level of speed and interest so as to make this important work readily available to the scientific community.

Sincerely,

James M. Gentile, Ph.D.
Editor-in-Chief
Mutation Research

City College of San Francisco
Advanced Technological Education for Biotechnology
National Center

23 January 2001

"To whom it may concern"
C/O Dr. James D. Tucker
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RE: R&D 100 application

Dr. Tucker and colleagues have made a significant step toward the identification of genes with mutations important to mammary cancer. At the same time they have developed a novel, fundamental tool for bridging the gap between cytogenetic and genomic analyses.

This novel invention, called gene recovery microdissection (GRM), allows us for the first time, to make customized chromosome region-specific cDNA libraries from any normal or diseased tissue of any plant or animal species. Use of GRM will permit researchers to determine the involvement of genes that are known, and those that are novel. Such an ability is critical to our understanding of gene expression and disease following chemical or physical and pharmacological or toxicological exposure. Additionally, this invention will permit us to map genes in those species with poorly characterized genomes, and identify genes in cross-species sytenic regions.

In my opinion, this discovery will find widespread, immediate use in the entire biotechnology industry.

Barton L. Gledhill, V.M.D., Ph.D.
Co-PI and Deputy Director

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Submission No. 422591

February 7, 2000 -- 12:58

[Commerce Business Daily: Posted in CBDNet on February 7, 2000]
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SUBJECT: A--LAWRENCE LIVERMORE NATIONAL LABORATORY SEEKS PARTNERSHIPS WITH INDUSTRY TO COMMERCIALIZE TECHNOLOGY FOR PRODUCING CHROMOSOME-REGION SPECIFIC GENE EXPRESSION LIBRARIES
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Mammalian Genome

PCR in situ followed by microdissection allows whole chromosome painting probes to be made from single microdissected chromosomes

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Received: 14 January 1999 / Accepted: 28 January 1999

Abstract. Whole-chromosome painting probes (WCPs) and chromosome-arm painting probes (CAPs) are an integral part of the cytogenetic analysis of chromosome abnormalities. While these are routinely made by chromosome microdissection, multiple copies of the dissected region have been necessary to achieve a library sufficiently complex to provide adequate painting. Performing multiple dissections of chromosomes or chromosome regions is time consuming and occasionally impossible, such as when working with species whose banded karyotype is not well defined. We have developed a method whereby chromosome paints can be reliably generated by dissecting single chromosomes. The technique consists of performing degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) in situ on the chromosomes, prior to dissection. Enough amplification occurs to enable a single dissected chromosome to be used to create a painting probe sufficiently complex for use in fluorescence in situ hybridization (FISH). The amplification products remain localized on the chromosomes; this allows region-specific chromosome paints to be made. We detail this novel technique and show whole-chromosome, arm-specific, and contiguous region-specific probes for human and rat, each created from single dissected fragments of chromatin.

Introduction

Microdissection has become a very popular method for making both whole-chromosome and region-specific painting probes (Guan et al. 1994a). With experience, the technique is rapid and efficient, and enables probes to be made from chromosomes whose size makes them difficult to separate by flow sorting. Microdissection can also be used to produce probes from such anomalies as marker and derivative chromosomes, making it a very powerful analytical technique (Guan et al. 1994b; Elkahloun et al. 1996).

Microdissection does possess one serious drawback, however. It is generally necessary to dissect multiple copies of a target chromosome or region to produce a probe sufficiently complex for FISH. Although band-specific probes have been made from single fragments (Guan et al. 1993), painting regions larger than this requires as many as 50 copies to be dissected for sufficient probe coverage; to date, no papers have been published in which whole-chromosome probes (WCPs) have been made with single dissected chromosomes. The need to dissect more than one copy of a target complicates the process of microdissection. For example, it can be difficult to locate precisely the same chromosomal region when making multiple scrapes of a single band. As a result, the painting probe covers a wider region than desired. When probes are made for adjacent bands, this can result in overlapping signals, which complicate analysis. Creating WCPs and Chromosome arm painting probes (CAPs) is also complicated by the need to dissect multiple chromosomes. Since many chromosomes, and indeed the p and q arms of metacentric chromosomes, are difficult to differentiate without banding analysis, the chromosomes must be banded before dissection. In the case of some genomes, such as mice, rats, or dogs, even banded chromosomes can be difficult to distinguish. Chromosomes from these species are also difficult to isolate by flow sorting because there is little difference in chromosome size. Since non-human mammalian models play an important role in cytogenetic research (Shi et al. 1997; Tucker et al. 1997), the ability to make chromosome paints from single scrapes of a band, arm, or chromosome is highly desirable.

In this paper we show that DOP-PCR performed in situ on target chromosomes prior to dissection increases the amount of DNA associated with a chromosome or chromosome region. This allows libraries complex enough to be used as FISH painting probes to be made from single dissected fragments. The coverage of the DOP-PCR in situ is broad enough to allow WCPs to be made, and specific enough to allow contiguous region-specific probes to be made from a single copy of one metaphase chromosome. We present WCPs, CAPs, and region-specific probes from humans and rats, generated with this technique, which we term HeadStart microdissection.

Materials and methods

Whole blood was cultured as previously described (Tucker et al. 1997; Johnson et al. 1998). At 48 h after culturing, Colcemid (Gibco BRL, Indianapolis, Ind.) was added to a final concentration of 0.1 μg/ml. The cultures were harvested 4 h later by treatment with hypotonic solution (0.075 M KCl) for 30 min at 37°C, followed by three fixations in methanol:acetic acid (3:1 vol/vol). The fixed cells were dropped onto 24 × 60 mm cover slips, air dried, and stored at room temperature.

Two cover slips were used for DOP-PCR in situ; one was used as a positive control by incorporating rhodamine-6-dUTP, and the other was used for microdissection. Fifty-μl reaction drops containing 5 μl Thermo Sequenase DNA Polymerase, 5 μl Thermo Sequenase reaction buffer (Amersham, Arlington Heights, Ill.), 200 μM of each dATP, dTTP, dCTP, and dGTP (Boehringer Mannheim, Indianapolis, IN), and 4 μM DOP primer (5′-CCGACTCGAGNNNNNNATGTGG-3′) were placed on unfrosted microscope slides; a cover slip was inverted and gently placed on the slide so that the cells were in contact with the solution. Rubber cement was used to seal the cover slip to the microscope slide. Control DOP-PCR in situ experiments included 40 μM tetramethylrhodamine-6-dUTP added to the reaction in addition to the components listed above. All PCR reactions were performed with a DNA Engine thermocycler (MJR Research Inc., Watertown, Mass.). The thermal profile consisted of 95°C for 10 min, 8 cycles at 94°C for 1 min, 30°C for 5 min, and a ramp of 0.1°C/s up to 65°C for 5 min, 12 cycles at 94°C for 1 min, 56°C for 5 min, and 72°C for 5 min, followed by 72°C for 5 min and held at 4°C until removed. Thermo Sequenase was used because of its stability at high temperatures; new polymerase need not be added following each cycle.

Once complete, the cover slips were removed from the slide and soaked in a 4 × SSC/0.1% triton solution for 5 min at room temperature. The fluorochrome-labeled cover slip was then mounted onto a microscope slide

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with 0.1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) and an antifade solution. Metaphase spreads were visualized with a Zeiss Axioskop (Carl Zeiss, Inc., Thornwood, N.Y.) and images captured by a Vysis QUIPS Imaging Analysis System (Vysis, Downers Grove, IL). The PCR was considered successful if all chromosomes in a metaphase spread were labeled with the fluorochrome.

The unlabeled cover slip was removed from the SSC solution, blown dry, and used for microdissection. Appropriately sized glass needles were made with a Flaming/Brown Micropipette Puller (Sutter Instrument Co., Novato, Calif.) With a Nikon Phase Contrast Microscope (Nikon Instruments Co., Melville, N.Y.) and a Narashige micromanipulator, one copy of the desired chromosome, arm, or region was scraped and placed in a 500-μl microfuge tube. The DNA was then amplified by PCR in a 15-μl reaction drop containing 1.5 μl Thermo Sequenase DNA Polymerase, 1.5 μl Thermo Sequenase reaction buffer, 200 μM of each dATP, dGTP, dCTP, and dGTP, and 4 μM DOP primer. Thirty microliters of mineral oil was added to the reaction mixture to prevent evaporation. The thermal profile consisted of 95°C for 10 min, 6 cycles of 94°C for 1 min, 30°C for 2 min, and a ramp of 0.1°C/s up to 65°C for 3 min, 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 3 min, followed by a single 72°C for 5 min and held at 4°C until removed. To verify that DNA amplification had occurred, we electrophoresed each sample on a 1.5% agarose gel at 100 V for 1 h. When appropriately sized products (300–600 base pairs) were identified, a fluorochrome was incorporated in a second-generation PCR with 2 μl of the first-generation product as a template. The 50-μl labeling reaction contained 20 U Thermo Sequenase DNA Polymerase, 26 mM Tris-HCl, pH 9.0, 6.5 mM $MgCl_2$, 200 μM of each dATP, dTTP, dCTP, and dGTP, 40 μM rhodamine-6-dUTP, and 4 μM DOP primer (5′-CCGACTCGAGNNNNNNATGTGG-3′). The thermal profile consisted of 95°C for 5 min, 25 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 3 min, followed by 72°C for 5 min and held at 4°C until removed.

The painting probe was added to a hybridization mix (50% formamide, 2 × SSC, 10% dextran sulfate, and 3 μg of blocking DNA) to a final volume of 15 μl. In the case of the rat, total genomic DNA was used as the blocking DNA, and CoT-1 DNA (Gibco BRL, Gaithersburg, MD) was used for the human hybridizations. Metaphase spreads were denatured in 70% formamide/2 × SSC, pH 7.0 for 3 min, followed by successive washes in 70%, 85%, and 100% ethanol for 3 min each. The probe mixture was denatured at 70°C for 5 min and applied to the denatured slides, covered with 22 × 22 mm cover slips, sealed with rubber cement, and hybridized overnight at 37°C in a 5% CO_2 atmosphere.

After hybridization, unbound probe was removed with three 5-min washes in 50% formamide, 2 × SSC, pH 7.0 (45°C), followed by one 5-min wash in 2 × SSC (45°C) and one 5-min wash in 2 × SSC with 1% Triton-X (45°C). The metaphase chromosomes were then counterstained with DAPI and a antifade solution. Metaphase spreads were observed with a Zeiss Axioskop fluorescence microscope with a dual band-pass filter for rhodamine and DAPI (Chroma Technology Corp., Brattleboro, VT), and images were captured by a Vysis QUIPS Imaging Analysis System.

Results

A rat metaphase spread after rhodamine-dUTP incorporation by DOP-PCR in situ is shown in Fig. 1. PCR in situ amplifications performed with rhodamine-dUTP but without primers showed little or no fluorescence associated with the chromosomes, whereas PCR in situ amplification performed with primers plus rhodamine-dUTP showed brightly labeled chromosomes. The localization of fluorescence around each chromosome indicates that DNA is being amplified during the PCR in situ reaction and that the nascent DNA remains associated with the chromosomes. The amplification protocol we used was based in part on the PCR in situ work of Gosden (Gosden et al. 1991; Gosden 1994; Gosden and Lawson, 1994a, 1994b) and the DOP-PCR work of Telenius (Telenius et al. 1992) and Guan (Guan et al. 1992). The initial eight cycles at a low annealing temperature were performed to produce DNA copies of the template that were properly sized for FISH (300–600 base pairs), with the DOP primer on both ends. The 12 high-annealing-temperature cycles were done to amplify exponentially the DNA fragments produced in the initial cycles.

Our primary concern with this technique was that the DNA products of the PCR in situ reaction would not remain closely associated with the regions from which they were replicated, an effect noted by Komminoth (Komminoth et al. 1992, Komminoth and Long 1993). This phenomenon is seen when doing many cycles of PCR in situ, wherein the PCR products diffuse away from the chromosomal region from which they were derived. This is a significant problem when attempting to localize a specific signal on a chromosome. Our initial efforts consisted of making WCPs for a variety of chromosomes, both human and rat, to ensure that only the desired chromosomes were labeled in subsequent FISH reactions. Figures 2A and 2B show WCPs for human Chr 1 and rat Chr 1, respectively, each made from a single chromosome on which DOP-PCR in situ had been performed prior to microdissection. None of the probes that we made cross-hybridized with any other chromosomes. We also dissected and pooled two, four, and six copies of human Chr 1 on which PCR in situ had been performed; each of the dissections produced probe, and there was no visible difference in probe coverage or intensity among the three products. None of the three probes differed from the probe made from a single chromosome in either intensity or coverage. Pooling chromosomes, as is usually done when microdissecting, is unnecessary with HeadStart microdissection.

We then attempted to determine the extent of product diffusion along the length of individual chromosomes. To accomplish this, we first made CAPs for various chromosomes to see if the probes labeled only single arms in the FISH reactions; a CAP for human Chr 1 is shown in Fig. 3A. Having determined that there was sufficient resolution to produce arm-specific paints, we then dissected a single copy of human Chr 2 in four contiguous pieces. Figure 3B shows the four adjacent probes, alternately labeled with FITC and rhodamine. These probes illustrate that diffusion of the DOP-PCR in situ products does not present a significant problem when dissecting regions adjacent to one another. It is possible, however, that product drift would present a problem for microdissection in situ if too many PCR cycles were done.

As a control experiment, microdissection followed by DOP-PCR was performed on three single chromosomes that had not been subjected to PCR in situ prior to dissection. None of the three dissected chromosomes produced a visible probe (data not shown). In contrast, the success rate of HeadStart microdissection with single dissected chromosomes is approximately 90%.

Recently, work by Engelen (Engelen et al., 1998) showed that hydrating chromosomes immediately prior to dissection makes them easier to life off the cover slip in one piece, rather than in fragments. This may result in greater chromatin recovery from each dissected chromosome; this would mean that fewer chromosomes would need to be dissected. Since the chromosomes become hydrated during the in situ PCR (but not, interestingly, any easier to life off the cover slip), we wanted to be sure that the increased efficiency of HeadStart microdissection was the result of DNA amplification, rather than simply hydration. We tested this by performing parallel dissections on two cover slips, immediately following PCR in situ. The same cycling protocol was done on both cover slips, but one of the reactions was done without primers. Fluorescent controls showed that rhodamine-6-dUTP had been incorporated in the reaction in which primers were present, but not in the primerless reaction. Three single copies of human Chr 1 were dissected from each of the two cover slips and put into separate microfuge tubes, and all six were subjected to DOP-PCR. Each of the three chromosomes dissected from the cover slip with the primer-containing PCR produced smooth paints that completely labeled Chr 1. None of the three probes made from the primerless cover slip produced complete paints, and only one labeled the target chromosomes at all. This paint was very spotty in appearance and could not be used to score aberrations.

Discussion

Numerous papers have been published demonstrating new techniques intended to make microdissection more rapid and efficient.

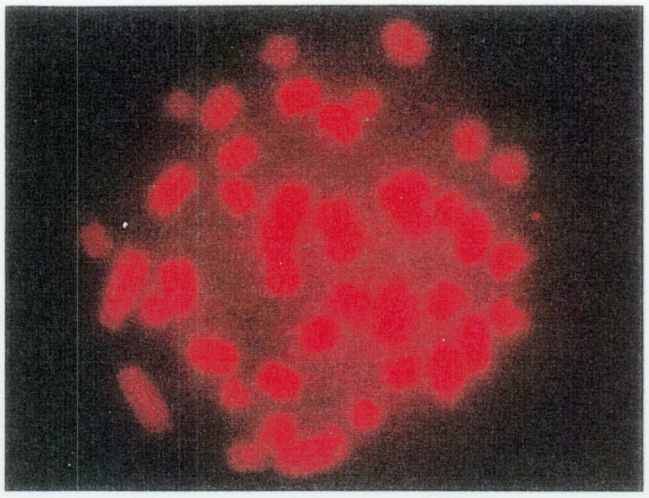

Fig. 1. A rat metaphase spread on which DOP-PCR in situ has been performed. The incorporation of rhodamine-6-dUTP allows the 'halo' effect characteristic of this process to be seen as a red fluorescent ring around each chromosome.

Through the use of new enzymes and better preparative methods, microdissection has become a widely used tool in cytogenetics. However, with one exception (Guan et al. 1993), researchers have found it necessary to dissect multiple copies of a target to produce quality probes.

There are several possible reasons why multiple chromosomes need to be dissected to make a library complex enough to be used for FISH. One reason is that it can be difficult to dissect an entire chromosome and be sure that all of the chromatin has been successfully transferred into a microfuge tube. Secondly, even if the entire chromosome is successfully transferred, the amount of DNA involved is very small. Slight preferences in primer annealing during the initial low-temperature PCR could produce substantial asymmetries during amplification, resulting in incomplete probe coverage. Engelen and associates (1998) were able to improve the efficiency of chromatin removal from the cover slip by hydrating the chromosomes prior to dissection. This additional step made the chromosomes easier to remove, presumably resulting in more complete transfer to the microfuge tube. However, even with this modification, multiple copies of the target chromosomes were dissected to produce the paints. HeadStart microdissection is unique in consistently allowing probes to be made from single dissected

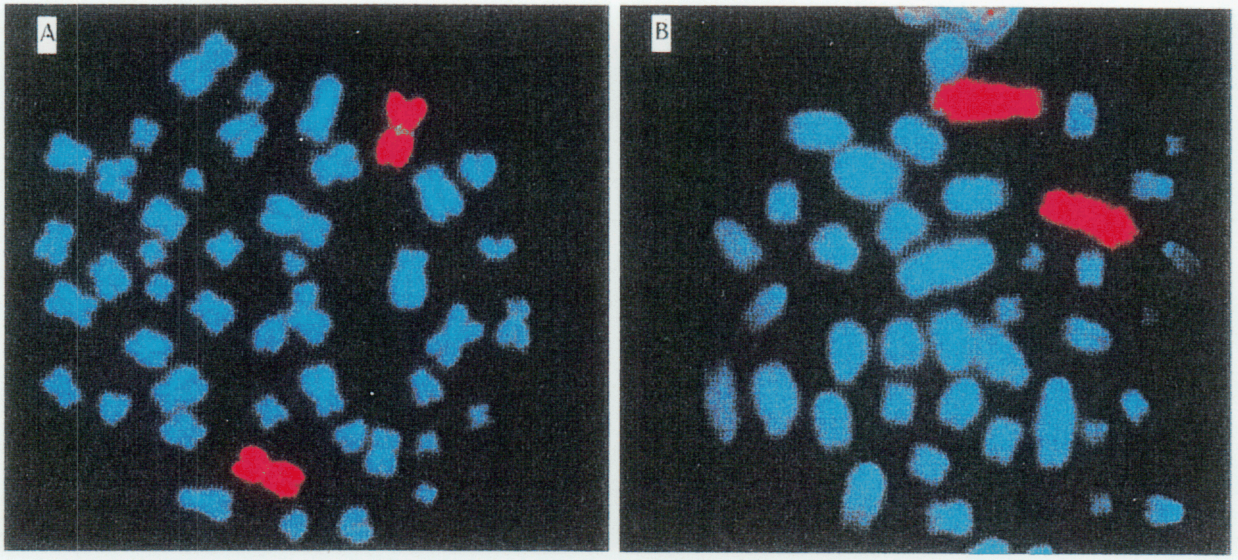

Fig. 2. Several painting probes made by dissecting single chromosomes or chromosome fragments following DOP-PCR in situ. **A:** A whole-chromosome paint for human Chr 1. **B:** A whole-chromosome paint specific for rat Chr 1.

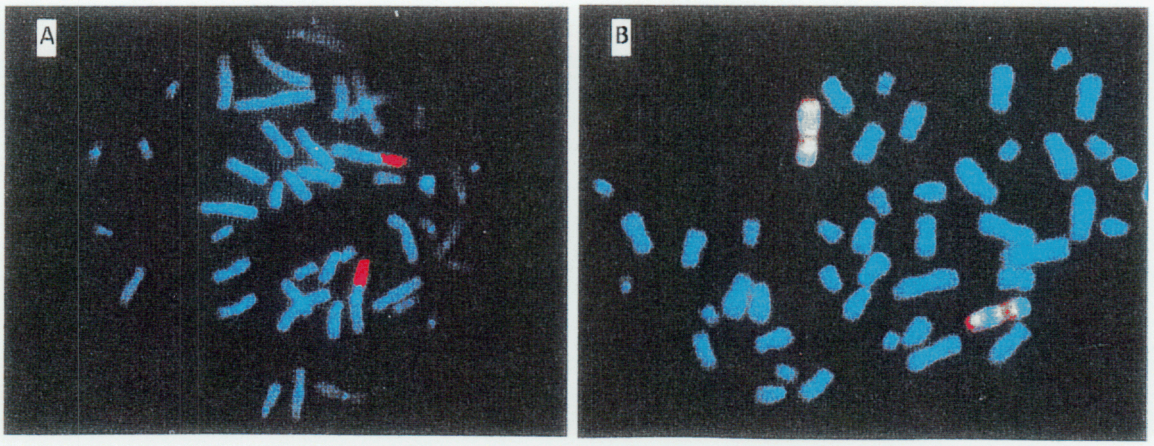

Fig. 3. Human CAP and region-specific probes. **A:** An arm-specific paint for human Chr 1. **B:** Paints for human Chr 2. A single copy of human Chr 2 was dissected in four scrapes, and each was labeled with either fluorescein-dUTP (green) or rhodamine-dUTP (red) and hybridized simultaneously.

chromosomes. This procedure differs slightly from the frequently used procedure developed (Guan et al. 1992), in that the DOP-PCR is performed on the cover slip prior to dissection and again in the microfuge tube after dissection. We believe that the PCR in situ increases the amount of DNA being added to the microfuge tube after dissection; further amplification in the tube then produces a more complex probe than would be possible if a single, unamplified chromosome were dissected.

The ability to create complex painting probes from single chromosome fragments has considerable utility in a variety of areas. One major advantage of HeadStart microdissection is a substantial decrease in the time required for dissection, reducing the cost of producing probes. The potential for contamination with foreign DNA is also reduced, since the microfuge tube needs to be opened only once.

A disadvantage of this technique is that chromosomes cannot be G-banded after DOP-PCR in situ. This makes dissecting of a particular chromosome difficult, unless it can be easily identified by morphology alone. However, since painting probes can be made so quickly with this technique, one can easily dissect several similar chromosomes and determine which is the correct one by banding analysis at the time of hybridization (Christian et al. 1998).

This technique will also make it easier to produce FISH painting probes from targets for which multiple dissections are difficult, if not impossible. Possible targets include maker and derivative chromosomes, clastogen-induced DNA breakpoints, the microchromosomes that are common in reptiles, amphibians, and birds, and the smaller chromosomes in mammalian genomes that are difficult to differentiate, such as rodents and dogs. HeadStart microdissection will substantially increase the utility of microdissection, making an already powerful technique even more useful.

Acknowledgments. The authors acknowledge Dr. Joel Bedford for his intellectual contribution to this technique. This work was performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract No. W-7405-ENG-48 with support from National Institutes of Health grant CA55861.

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Analyses of PhIP-Induced Mammary Carcinomas in Rats Reveal a Possible Cytogenetic Signature

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This work was performed under the auspices of the U.S. Department of Energy by the University of California, Lawrence Livermore National Laboratory under Contract No. W-7405-Eng-48.

ABSTRACT

PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine), a mutagen/carcinogen belonging to the class of heterocyclic amines (HCAs) found in cooked meats, is a mammary gland carcinogen in rats and has been implicated in the etiology of certain human cancers including breast cancer [1-2]. To gain insight into the genomic alterations associated with PhIP-induced mammary gland carcinogenesis, we used comparative genomic hybridization (CGH) to examine chromosomal abnormalities in rat mammary carcinomas induced by PhIP, and for comparison, by DMBA (7, 12-dimethylbenz[a]anthracene), a potent experimental mammary carcinogen. We found a consistent and characteristic pattern of chromosome-region loss in PhIP-induced carcinomas that clearly distinguished them from carcinomas induced by DMBA. Of six PhIP-induced mammary gland carcinomas examined by CGH, all showed losses in the same specific regions of chromosomes 2, 3, 11, 18, and X. With the long-term goal of determining which genes in these common deleted regions are involved in PhIP-induced mammary carcinogenesis, we developed a method to generate chromosome region-specific cDNA libraries, referred to as gene recovery microdissection (GRM). To confirm the utility of GRM, expressed genes at rat chromosome 1p12-q31 were isolated, mapped and shown to correspond to the known syntenic regions in human and mouse chromosomes. We then used GRM to recover known and novel genes from the affected regions of chromosomes 2 and 3. Among the genes isolated were tumor suppressor genes and various regulatory genes, which will provide targets for future study on the genetic mechanisms of PhIP-induced mammary carcinomas. The GRM approach is generally applicable for studies of species with incompletely characterized genomes, such as the rat, and will help to bridge the gap between cytogenetic and genomic analyses.

INTRODUCTION

Human breast cancer is characterized by multiple genomic alterations and many of the critical genes involved in this disease remain to be elucidated [3-4]. The etiology of breast disease is not entirely understood, however, dietary factors may impact breast cancer risk [2, 5-6]. PhIP is a heterocyclic amine food mutagen found in the human diet that is produced during the cooking of meats. It has been shown to be a potent rodent mammary gland carcinogen [7-9], and PhIP has recently been shown to be associated with a higher risk of breast cancer in women [2, 5].

Mammary carcinogenesis in rats is recognized as a valuable model for the human disease [4] [10, 11]. Carcinomas in rats and humans are similar in the site of origin from ductal elements, the susceptibility of the mammary gland to initiation during development (i.e., period of adolescence), and the hormonal responsiveness of the tumors [10]. Studies using the rat mammary cancer model to study environmental carcinogens such as PhIP may provide a view to the specific genomic alterations of the disease, and of the role of specific carcinogens in the etiology of human breast cancer.

We examined by CGH mammary carcinomas that were induced either by the food mutagen PhIP or DMBA, a potent experimental mammary gland carcinogen belonging to the class of polycyclic aromatic hydrocarbons, in female Sprague-Dawley rats. Although routinely applied to studies of the genomic alterations in mouse and human tumors, CGH has not been widely utilized in studies of the rat genome. It is used to detect genomic changes such as losses, gains and amplifications of chromosomes and chromosome regions, and can provide valuable information about solid tumors from which metaphase cells are difficult to obtain [12-14]. We examined 9 carcinomas, 6 induced by PhIP and 3 induced by DMBA. All 9 of the carcinomas contained genomic changes detectable by CGH as either losses or gains/amplifications of chromosomal regions.

Since the rat genome has not yet been mapped to the extent of humans or mice, it is currently difficult to determine which genes in these deleted regions might be important for tumorigenesis. Thus, another means must be used to identify the known or novel genes in these regions that are expressed in normal rat mammary tissue. We have developed gene recovery microdissection (GRM), a technique that combines HeadStart microdissection [15] with a process variously referred to as preparative in situ hybridization [16] or microdissection-mediated cDNA capture [17, 18]. We demonstrate proof-of principle of the GRM technique and present results from 2 of the 5 regions of common deletion observed in PhIP-induced mammary carcinomas, which are rat chromosomes 2 and 3.

MATERIALS AND METHODS

Mammary Tumor Samples: PhIP-induced carcinomas were obtained from a previous study in which tumors developed over a 25-week period after administration of PhIP (75 mg/kg, p.o., once per day, for 10-days) or DMBA (10mg/kg, p.o., single dose) to 50-day old rats [8, 9]. Rats were placed on a defined high-fat or low-fat diet after carcinogen treatment [8]. Vehicle control animals were run in parallel in both the PhIP and DMBA studies, and none of the control rats developed mammary carcinomas during the 25-week study [9]. This technique, based on the model developed by Huggins, produces cancers only in animals treated with the specific agent, avoiding spontaneous tumors that can complicate analyses [19, 20]. All carcinomas used in this study were tubular or tubulopapillary carcinomas as classified by the criteria of Russo and colleagues [10, 11]. DNA isolated from the tumors was amplified and labeled by PCR in a 15 µl reaction drop containing 1.5 µl Thermo Sequenase DNA Polymerase, 1.5 µl Thermo Sequenase reaction buffer, 200 µM of each dATP, dGTP, dCTP, and dGTP, 2mM labeled nucleotide (either lissamine or fluorescein dUTP) and 4 µM DOP primer. The thermal profile consisted of 95°C for 10 min, 6 cycles of 94°C for 1 min, 30°C for 2 min, and a ramp of 1°C/s up to 65°C for 3 min, 20 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 3 min, followed by a single 72°C for 5 min and held at 4°C until removed.

CGH: Equal masses (~ 100 ng) of reference (normal) DNA and test (tumor) DNA are co-hybridized to normal metaphase spreads in the presence of ~ 50 µg of unlabeled CoT-1 repetitive-sequence blocking DNA. The DNA was added to a hybridization mix (50% formamide, 2 X SSC, 10% dextran sulfate) to a final volume of 15 µl. Metaphase spreads were denatured in 70% formamide/2 X SSC, pH 7.0 for 3 min followed by successive washes in 70%, 85% and 100% ethanol for 3 min each. The probe mixture was denatured at 70°C for 5 min and applied to the

denatured slides, covered with 22 X 22 mm coverslips, sealed with rubber cement and hybridized overnight at 37°C in a 5% CO_2 atmosphere. Following hybridization, unbound probe was removed with three 5-min washes in 50% formamide, 2 X SSC, pH 7.0 (45°C), followed by one 5-min wash in 2 X SSC (45°C) and one 5-min wash in 2 X SSC with 1% Triton-X (45°C). The metaphase chromosomes were then counterstained with DAPI and an antifade solution. Metaphase spreads were observed using a Zeiss Axioskop fluorescence microscope and images were captured by a Vysis QUIPS Imaging Analysis System (Vysis, Downers Grove, IL).

cDNA library manufacture: Rat mRNA was isolated from rat mammary tissue using the Ambion Poly(A)Pure mRNA isolation kit (Catalog #1915, Ambion Inc., Austin, TX). Human testis mRNA was purchased from Clontech Industries (Palo Alto, CA). First strand cDNA synthesis was done using the Amersham cDNA synthesis module (Catalog #RPN1256, Amersham Life Science, Buckinghamshire, England). The first strand primer was a modified version of the Life Technologies 3' RACE primer (Life Technologies, Rockville, MD), with the addition of a random dATP, dGTP or dCTP on the 3' end to serve as an anchor. First strand synthesis time was cut to 15 minutes to provide a shorter library that was more amenable to hybridization. The sample was treated with RNase H per the Amersham kit, and a poly dC tail was added using terminal transferase (Roche, Indianapolis, IN). Twenty cycles of PCR were done using the Life Technologies Abridged Universal Amplification Primer (AUAP, 20 nM), 5 µl Thermo Sequenase DNA Polymerase, 5 µl Thermo Sequenase reaction buffer, 200 µM of each dATP, dGTP, dCTP, and dGTP in a 50 µl reaction volume. The thermal profile consisted of 95°C for 10 min, 20 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 3 min, followed by a single 72°C for 5 min and held at 4°C until removed.

DOP-PCR in situ: Fifty-µl reaction drops containing 5 µl Thermo Sequenase DNA Polymerase, 5 µl Thermo Sequenase reaction buffer (Amersham, Arlington Heights, IL), 200 µM of each dATP, dTTP, dCTP, and dGTP (Boehringer Mannheim, Indianapolis, IN), and 4 µM DOP primer (5'-CCGACTCGAGNNNNNNATGTGG-3') were placed on unfrosted microscope slides; a coverslip was inverted and gently placed on the slide so that the cells were in contact with the solution. Rubber cement was used to seal the coverslip to the microscope slide. All PCR reactions were performed using a DNA Engine thermocycler (MJR Research Inc., Watertown, MA). The thermal profile consisted of 95°C for 10 min, 8 cycles at 94°C for 1 min, 30°C for 5 min, and a ramp of 0.1 °C/s up to 65°C for 5 min, 12 cycles at 94°C for 1 min, 56°C for 5 min, and 72°C for 5 min, followed by 72°C for 5 min and held at 4°C until removed. Once complete, the coverslips were removed from the slide and soaked in a 4 X SSC/0.1% triton solution for 5 min at room temperature.

cDNA in situ Hybridization: Forty-µl reaction drops containing 50% formamide, 2xSSC, 10% dextran sulfate, 10 µg CoT-1 blocking DNA, and approximately 100 ng cDNA were added to the PCR in situ-amplified coverslips, which were then placed on glass microscope slides and sealed with rubber cement. The rubber cement was allowed to dry, and then the slides were heated to 75°C for 15 min to denature probe and target, and incubated for 48 h at 37°C. The slides were washed in 2xSSC for 15 min at 42°C, rinsed with distilled water and blown dry with nitrogen.

cDNA in situ PCR: Fifty-µl reaction drops containing 5 µl Thermo Sequenase DNA Polymerase, 5 µl Thermo Sequenase reaction buffer (Amersham, Arlington Heights, IL), 200 µM of each dATP, dTTP, dCTP, and dGTP (Boehringer Mannheim, Indianapolis, IN), and 5 µM AUAP primer were placed on an unfrosted microscope slide; the coverslip was inverted and gently placed on the slide

so that the cells were in contact with the solution. Rubber cement was used to seal the coverslip to the microscope slide. The thermal profile consisted of 95°C for 10 min and 6 cycles of 94°C for 2 min, 56°C for 5 min, 56°C for 5 min and a 5 min incubation at 72°C, and held at 4°C until removed. Once complete, the coverslips were removed from the slide and soaked in a 4 X SSC/0.1% triton solution for 5 min at room temperature.

Post-dissection Amplification: Fifty cycles of PCR were done in 15 µl volumes containing 20 nM AUAP, 1.5 µl Thermo Sequenase DNA Polymerase, 1.5 µl Thermo Sequenase reaction buffer, 200 µM of each dATP, dGTP, dCTP, and dGTP. The thermal profile consisted of 95°C for 10 min, 50 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 3 min, followed by a single 72°C for 5 min and held at 4°C until removed.

RESULTS AND DISCUSSION

All of the PhIP-induced carcinomas had five common regions of chromosome loss (Figure 1). However, no common chromosomal abnormalities were observed among the DMBA-induced carcinomas, nor were there any common abnormalities between the DMBA- and the PhIP-induced cancers (data not shown). The characteristic cytogenetic signature for PhIP-induced carcinomas made them clearly distinguishable from the carcinomas induced by DMBA. Besides the losses in common, each PhIP-induced carcinoma also possessed many other cytogenetic alterations.

Since the resolution of CGH is ~10-15 megabases, clearly not all abnormalities in these mammary carcinomas are likely to have been detected. However, the five common deletions that represent a signature pattern for PhIP-induced carcinomas indicate regions harboring genes in which a partial or complete loss of function, such as tumor suppressor genes, may play a role in PhIP-induced mammary carcinogenesis. Consistent with our CGH findings, PhIP-induced carcinomas also show a high frequency of allelic imbalance, which is not observed in DMBA-induced carcinomas [21, 22]. As indicated by our data and results from other studies, multiple genetic alterations play a role in carcinogenesis [23]. However, little is known about the genomic alterations associated with the development of rat mammary gland cancer by PhIP or other agents. The cytogenetic signature may be akin to a DNA 'fingerprint', or characteristic mutation, which has been linked to several agents including PhIP and can be found in the genome of tumors arising after carcinogen exposure [24]. For example, studies have shown that PhIP-induced colorectal cancer in rats may harbor a characteristic frameshift mutation in the Apc gene [25]. Our findings are novel in extending the molecular signature of PhIP in the mammary gland to the cytogenetic level. The finding that characteristic cytogenetic alterations are induced in PhIP-induced mammary gland cancer has implications for discovering which genes may be associated with PhIP-induced rat

mammary gland carcinogenesis and for linking exposure to an environmental chemical carcinogen to a specific human cancer.

To bridge the gap between cytogenetic and genomic analyses, we developed Gene Recovery Microdissection (GRM) to make chromosome region-specific cDNA libraries. GRM can be used with any species and tissue, and requires microdissecting only one normal metaphase chromosome, eliminating the need to obtain multiple copies of the target. The process, shown in Figure 2, involves hybridizing a cDNA library specific for the species and tissue of interest onto DOP-PCR amplified metaphase chromosomes. This step also normalizes the library, increasing the ratio of less prevalent to more prevalent expressed genes [26]. PCR is used again to amplify the cDNA molecules in situ following hybridization, and the desired chromosome regions are isolated by microdissection. The cDNA molecules hybridized to the dissected genomic DNA are then amplified by PCR in a tube by using primers specific for the linker arms on the cDNA. The cDNA molecules are then cloned and sequenced. In situ amplification followed by microdissection allows complex libraries to be made from single microdissected chromosomes and chromosome regions, possibly by increasing both the number of targets for in situ hybridization and probe accessibility to the target chromosomes. Hybridization of a 150 kb BAC to target chromosomes that had been amplified using DOP-PCR in situ resulted in a significant increase in signal intensity over similar hybridizations to unamplified target chromosomes (data not shown).

To test the efficacy of this technique, we performed the in situ amplifications and hybridized a human testis cDNA library to normal human metaphase chromosomes on which DOP-PCR in situ had been performed. Following hybridization, we amplified the bound probe with linker-specific PCR in situ and dissected the q-terminal band of human chromosome 2. After PCR amplification of the cDNA library using the linker primers, we used a primer set specific for the 3' end of the *HHARP* gene, which had been mapped to this region previously [27] (Figure 3a). We dissected 6 q-

terminal fragments, amplified them separately, and were able to amplify the *HHARP* gene from each of them. As a control to check PCR product drift on the slides, the neighboring band was also dissected from each chromosome; the *HHARP* gene PCR products were not seen in any of these scrapes (Figure 3b). This supported earlier results, in which contiguous genomic DNA libraries made by microdissection following DOP-PCR in situ were shown to have little to no overlap [15].

We then performed GRM using a rat mammary tissue cDNA library, hybridized onto normal rat metaphase cells. We focused on the rat chromosome 1 (RNO1) for our initial studies. RNO1 has known synteny to human chromosomes 5, 6 and 19 and mouse chromosome 7, all of which have been well characterized, making this an ideal test chromosome. We picked and sequenced (forward and reverse) 192 clones from a region spanning approximately RNO1p12-q31, and performed homology searches using the DataFoundry database storage and mining system [28]; results are shown in Figure 4a. Many of the hits were novel sequences; of those that had database homology to known genes, none had been mapped in the rat. However, many of the known genes that we sequenced have been mapped to syntenic regions in humans and mice, confirming the utility of GRM for recovering genes in selected chromosome regions. In addition, we isolated a BAC with a randomly selected clone; the BAC hybridized to the dissected region of RNO1 (Figure 4b).

Next, we microdissected regions from rat chromosomes 2 and 3 that corresponded to the deleted regions in the PhIP-induced carcinomas. Our hope was that tumor suppressor candidate genes might be located in these regions, providing us with potential targets for further analysis. Results are shown in Figure 5, and indicate that several candidate genes may reside in these regions.

The goal in developing GRM was to develop a comprehensive list of possible tumor suppressor genes involved in PhIP-induced rat mammary carcinogenesis by isolating all of the genes expressed in rat chromosomal regions that show recurrent loss in the PhIP carcinomas. Knowledge of the number of genes expressed in each region in rat mammary tissue would permit a simple power

analysis to be applied to statistically estimate the number of post-dissection clones required to ensure that the desired fraction of expressed sequences from each dissected fragment was isolated and sequenced. However, there is considerable disagreement regarding both the number of genes in a "typical" mammalian genome and the percentage of those genes expressed in a given tissue. Without knowing how many expressed genes we could expect to see in a particular region, we decided instead to develop a picture sufficient to facilitate further analysis of genes potentially involved in mammary carcinogenesis. Subsequent work will be done in collaboration with the Joint Genome Institute to determine the number of genes expressed in rat mammary gland in each chromosomal region. Future analyses will also include screening and cytogenetically mapping the many sequence matches that were returned from database searches.

Applying the GRM technique to the regions of rat chromosomes 2 and 3 that showed recurrent loss in the PhIP-induced carcinomas, several tumor suppressor genes were putatively located (Figure 5), providing potential candidates for further studies on their impact on mammary carcinogenesis. Although the orthologous regions between rat and human have not been completely confirmed, it is notable that these deletions located on rat chromosomes 2, 3, 11, 18, and X are potentially orthologous to regions of human chromosomes 5q, 11p, 3p, 18q, and X, respectively, which harbor deletions (detected by LOH or CGH analysis) in certain human breast cancers [3, 29-31]. The short arm of chromosome 11, for example, is lost in 30% of human breast cancers [32], and contains the putative tumor suppressor gene tsg101, which we have tentatively shown to be mapped to RNO 3. This gene was shown to contain intragenic mutations in 7 of 15 primary human breast carcinomas that showed LOH in 11p [33].

In summary, we report recurrent regions of loss by CGH in PhIP-induced rat mammary carcinomas by CGH analysis. In addition, we describe a microdissection method which will permit detailed genetic mapping of the rat chromosomal regions and facilitate the identification of critical

genes associated with PhIP-induced rat mammary gland carcinogenesis. Comparison between rat and human mammary cancers is expected to ultimately aid in further understanding the genetic changes responsible for mammary cancer induction with a dietary carcinogen.

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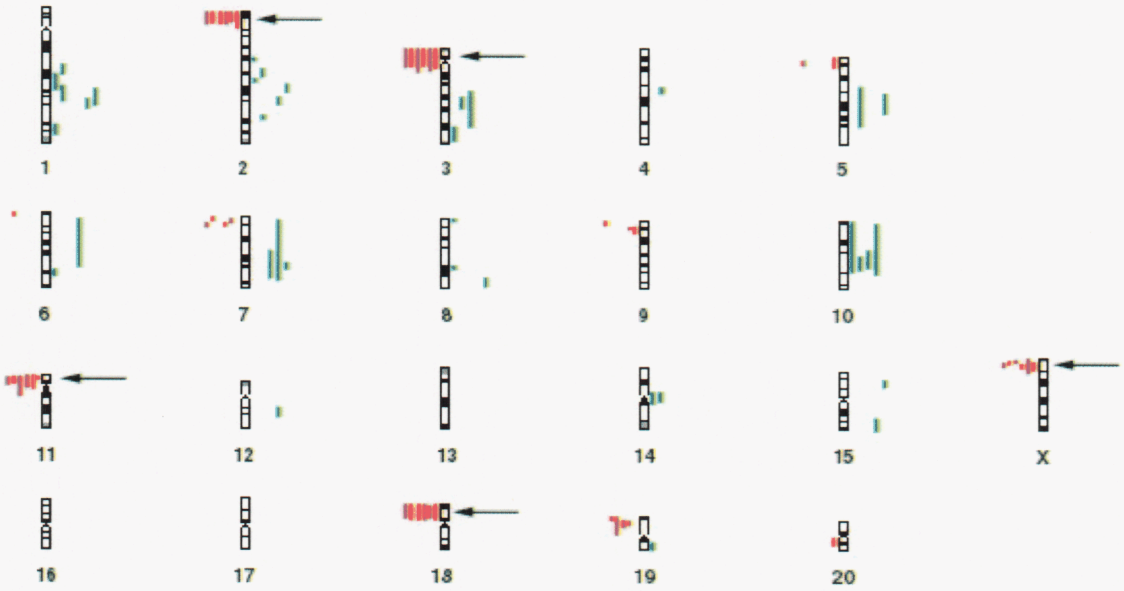
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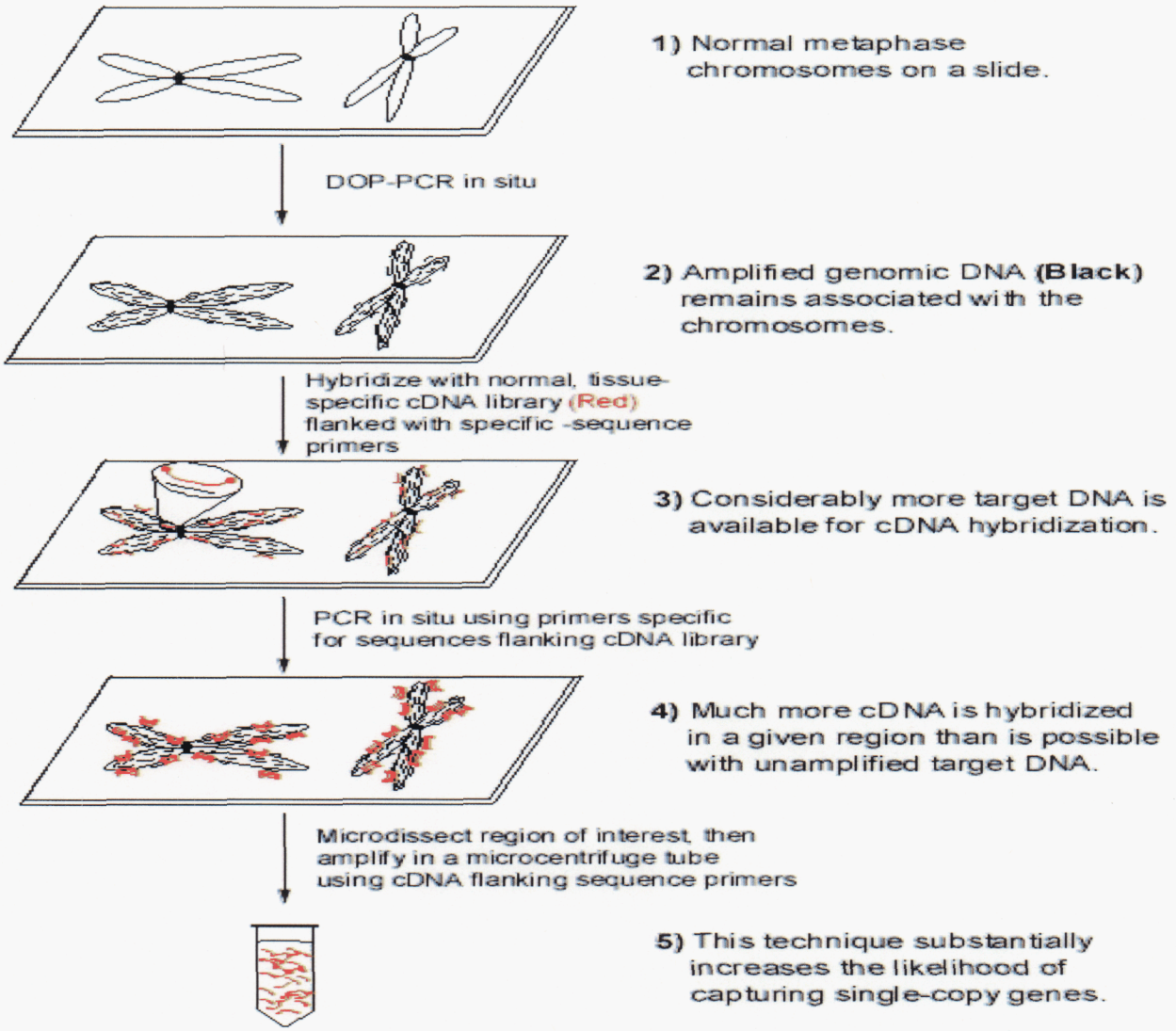
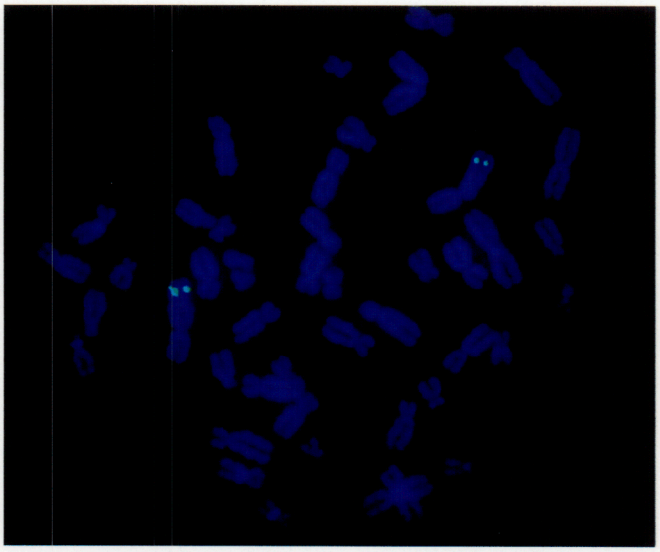
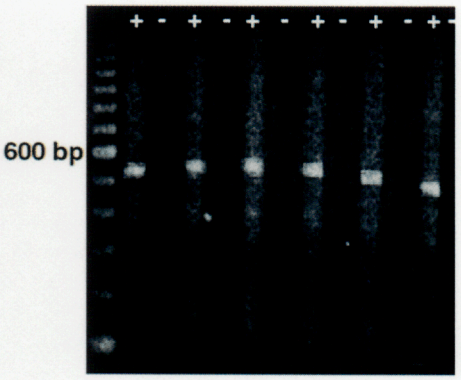

1) Normal metaphase chromosomes on a slide.
DOP-PCR in situ
2) Amplified genomic DNA (Black) remains associated with the chromosomes.
Hybridize with normal, tissue-specific cDNA library (Red) flanked with specific -sequence primers
3) Considerably more target DNA is available for cDNA hybridization.
PCR in situ using primers specific for sequences flanking cDNA library
4) Much more cDNA is hybridized in a given region than is possible with unamplified target DNA.
Microdissect region of interest, then amplify in a microcentrifuge tube using cDNA flanking sequence primers
5) This technique substantially increases the likelihood of capturing single-copy genes.





a *b*

Clones assigned a putative function based on homology

Clone	Putative Function	BLAST Score
F12	Translational tumor protein p21	97% ID
E09	Type III collagen precursor	30% ID
F07	Serine/threonine kinase	41% ID

Clones with only EST homology (100% match with existing rat EST sequences)

Clone	Identifier	Blast Score
E04	EST251684	652 Evalue 0.0
E10	EST204705	767 Evalue 0.0
E11	EST251684	317 Evalue 2.0E-84
F03	EST1749411	837 Evalue 0.0
F10	EST2418415	119 Evalue 1.0E-24
F11	EST2418415	180 Evalue 4.0E-43

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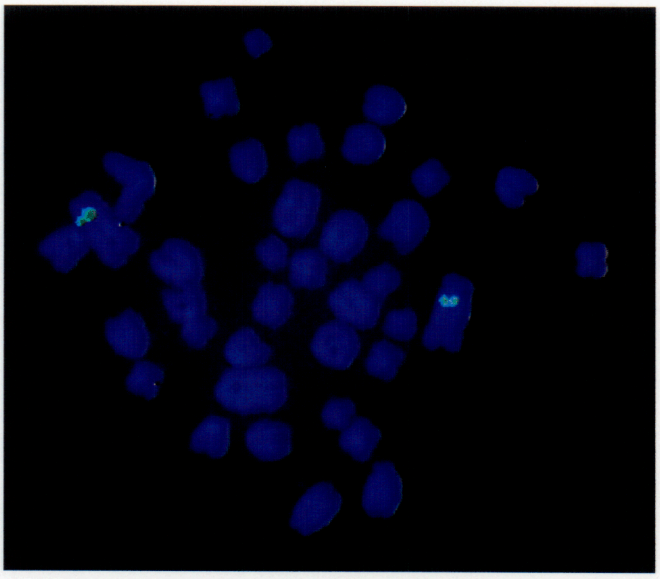

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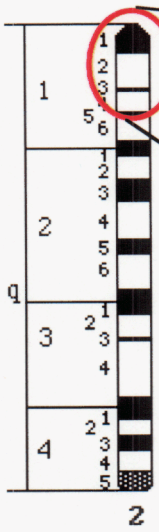
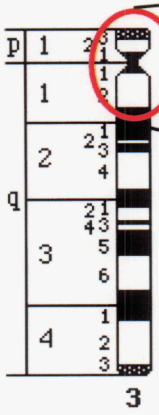


Unordered Genes Identified (Chromosome 2)	
Clone	Putative Function Based on Homology
B03	Rat Ribosomal protein L23A 100%id
B12	Hypothetical protein ligase-like 71% id.
A06	Cysteine rich mbn protein 68% id.
A03	ADP/ATP Carrier 30% id.



Unordered Genes Identified (Chromosome 3)	
Clone	Putative Function Based on Homology
3-24	Rat RNA Helicase P47 88% id.
3-25	Tumor Susceptibility Gene tsg101 100% id.
3-18	Zfp96 zinc finger protein 93% id.

Rat Ideograms from
ratmap.gen.gu.se

FIGURE LEGENDS:

FIGURE 1. Comparative Genomic Hybridization: CGH data pooled from 6 tumors induced by PhIP are shown. Green bars to the right of the ideograms show regions of DNA gain in tumors; red bars to the left show losses. Gain/loss bars closest to the ideogram represent the first tumor, gain/loss bars for the sixth tumor are the farthest from the ideogram. Note the five regions that are consistently lost (short arms of chromosomes 3, 11 and 18, and the centromeric regions of chromosomes 2 and X), indicated by arrows.

FIGURE 2, Gene Recovery Microdissection: cDNA libraries were constructed for the tissue of interest; the end result was a library with a median size of approximately 800-1200 base pairs with linker sequences attached to each end. Prior to hybridizing the cDNA to metaphase spreads, the spreads underwent extensive preparation. Normal metaphase cells first underwent *in situ* DOP-PCR (Step 1) to amplify the chromosomal (target) DNA on the slides. Following washing to remove nonspecifically bound DNA, the slides are crosslinked in a Stratalinker 1800 (Stratagene, LaJolla, CA), to preclude PCR amplification of the genomic DNA. Next, the cDNA library was hybridized to these cells (Step 2). This hybridized library was amplified *in situ* using primers specific for the flanking linkers used in the library synthesis (Step 3). The genomic regions of interest containing the hybridized cDNA are microdissected and the individual dissected fragments are placed in microcentrifuge tubes, and the cDNA molecules are amplified again with the linker-primed PCR (Step 4). The resulting chromosome-region specific cDNA libraries were sequenced at the Joint Genome Institute.

FIGURE 3, Precision and Repeatability of GRM: 3a shows a Bacterial Artificial Chromosome containing the human *HHARP* gene mapped to human chromosome 2. 3b shows results from dissecting 6 different human chromosomes 2 following the complete GRM procedure, in which a human testis cDNA library containing the *HHARP* gene was hybridized to normal human chromosomes. In each of the 6 cases, the region containing the mapped gene was dissected, as was the immediately contiguous region in which the gene was ostensibly not present. Each of the 12 dissected regions (6 presumably containing the *HHARP* gene, and 6 not) were amplified separately using PCR primers specific for the flanking primers of the cDNA library for 50 cycles, then with primers specific for a ~550 base region of the *HHARP* gene for 35 cycles. Following the 85 cycles of PCR, the products of each reaction were run on an agarose gel, with each gene-containing region being run next to its gene-lacking contiguous counterpart. In all 6 cases, the gene was amplified from the region to which it mapped, but not from the contiguous region (every other lane contains a product; these lanes represent regions from which the gene-containing fragment was dissected). This illustrates both the sensitivity and precision of GRM.

FIGURE 4, Genes Putatively Mapped to Rat Chromosome 1: The sequenced clones were searched via nucleotide sequence and protein search engines sponsored by NIH, including BLAST and dBEST to find EST (Expressed Sequence Tag) clones and non-redundant nucleic and protein sequences with high homology to human, mouse or known rat genes. Searches were performed using the DataFoundry search engine. Data from mapping of the rat and human genes was further compared using NCBI's Homology Mapping web site www.ncbi.nlm.nih.gov/Homology/) and http://ratmap.gen.gu.se/. Figure 4A shows the microdissected region of rat chromosome 1(red oval) and the genes that have been putatively mapped to that location. Putative functions are based on

homologies as shown. In addition, numerous other clones were unique, containing no known homologies. Figure 4B shows the FISH results for a BAC isolated using a randomly selected cDNA clone isolated from rat chromosome 1. The BAC is hybridized to the region of the chromosome 1 that was dissected.

FIGURE 5, Genes Putatively Mapped to Rat Chromosomes 2 and 3: Red ovals show the approximate regions that were isolated by microdissection.

The Generation and Utilization of Tissue- and Chromosome Region-Specific Gene Expression Libraries

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We have developed a process to make gene expression libraries from specific regions of chromosomes, using any tissue, normal or cancerous, for any species of animal. Using this technique, the involvement of known genes in various diseases can be determined, as well as the potential involvement of expressed novel genes. This technology can also be used to map genes in species whose genome is less well-known than the human genome by isolating cDNA from a particular region and comparing those sequences against the human expressed sequence tag database.

We have combined this technique with comparative genomic hybridization (CGH) to identify and map genes to chromosomal regions that are ubiquitously deleted in rat tumors. In rat mammary carcinomas induced by the heterocyclic amine PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine), CGH results show that five regions of the genome are consistently lost. Using the microdissection gene recovery technique to isolate cDNA from the coextant regions in normal rat metaphase spreads, we have sequenced numerous genes of interest from two of these regions, including DNA repair and tumor suppressor genes that were not previously mapped in the rat. We have also sequenced novel genes whose function is as yet unknown.

This technique represents a powerful new way to analyze the response of both known and novel genes to a specific chemical or physical agent. Once the expressed sequences from a region have been isolated, the libraries can be placed on 'chip arrays' and used to assay the presence, absence and differential expression levels of genes from specific chromosomal regions. As an added benefit, gene location on a chromosome can be mapped easily. cDNA from one species can also be hybridized to the chromosomes of another species, allowing synteny to be established between them. This provides a valuable means of linking data from animal models to the human genome. When coupled with the ever-growing database of genomic sequence information, this technique will have a large impact on the use of expressed sequence data in the fields of carcinogenesis and genetic toxicology.

This work was performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract No. W-7405-ENG-48 with support from NIH grant CA55861.